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Lidamycin inhibits tumor growth and pulmonary metastasis in murine breast carcinoma and shows synergy with paclitaxel

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The main goal of this study was to investigate whether lidamycin (LDM) could enhance the efficacy of paclitaxel (TAX) against breast cancer. In the MTT assay, LDM showed much more potent cytotoxicity than paclitaxel, and there was a synergy on the 4T1/luc breast cancer cells treated with a combination of paclitaxel and LDM. Western blot analysis showed that paclitaxel and LDM synergistically downregulated MMP9, MMP2, VEGF, and upregulated the cleaved PARP proteins. By wound closure cell migration assay, paclitaxel combined LDM obviously inhibited the migration of 4T1/luc cells. At therapeutic dosage level, LDM, paclitaxel, and the combination suppressed the pulmonary metastases by 70.2%, 53.8%, and 88.7%, respectively, and the CDI value was 0.82, indicating synergism. The results show that LDM enhances the antitumor effect of paclitaxel on 4T1/luc breast cancer, in particular, the antimetastatic effects on pulmonary metastasis.

lidamycin, paclitaxel, breast cancer, antitumor, antimetastatic

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Breast cancer has been the second leading cause of cancer-related mortality among women throughout the world [1]. Surgery is still the mainstay of treatment for primary breast cancer, but is ineffective for metastatic tumors at distant sites, and it may paradoxically increase the dissemination of tumor cells into the bloodstream resulting in the seeding of tumor cells in distant organs [2,3]. Adjuvant chemotherapy for breast cancer after surgery has been the standard of care, and can effectively prevent the occurrence of tumor cell dissemination and metastasis. Paclitaxel (Taxol, TAX) has been proved to be a potent drug to treat metastatic breast cancer, ovarian cancer, and other forms of cancer [4]. It blocks the cell cycle in the G2 and M phase by preventing polymerization of microtubules, thereby causing toxicity and cell death [5]. Paclitaxel is active in the treatment of metastatic breast cancer as first-line therapy [6], as well as in heavily pretreated patients [7,8]. Especially encouraging is its activity in anthracycline-resistant disease [9]. However, several toxic side effects and clinical implications, including severe anaphylactic peripheral neuropathy and hypersensitivity reactions, have been reported in paclitaxel-treated patients. Therefore, potential therapeutic strategies to improve the efficacy of paclitaxel and reducing its side effects are needed.

Anticancer antibiotic lidamycin (LDM, C-1027) was screened from the broth filtrate of *Streptomyces globisporus* C-1027 by spermatogonial assay. Strain C-1027 was isolated from a soil sample collected in Qianjiang area, Hubei Province of China [10]. LDM belongs to a family of enediyne antibiotics and shows extremely potent cytotoxicity by causing DNA double-strand breaks. LDM also markedly inhibits the growth of transplantable tumors *in vivo* [11–13]. LDM has demonstrated unique characteristics in the treatment of cancer, such as preferentially targeting hypoxic cells, dosage-dependent regimens based upon p53 status, and high activity against multidrug-resistant cancer cells [14–16]. LDM is now in phase II clinical trials as a potential chemotherapeutic agent in China.

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Recent study revealed that LDM combined with other anticancer drugs can effectively enhance the antitumor activity [17,18]. In this study, the highly metastatic 4T1 breast cancer model was used. As well known, the 4T1 cell line was derived from a spontaneously arising BLAB/c mammary tumor. It grows progressively and causes a uniformly lethal disease even after excision of the primary tumor [19]. 4T1 tumor metastasizes via the hematogenous route to the liver, lungs, bone, and the brain. It closely resembles stage IV human breast cancer both in its immunogenicity, growth and metastatic properties, making it an excellent animal model to evaluate the effects of drugs on breast tumor metastases as well as tumor growth [20–22]. Herein, we investigated whether the combination of LDM with paclitaxel would enhance the antitumor and antimetastatic activity to combat the highly metastatic 4T1 breast cancer *in vitro* and *in vivo*.

1 Materials and methods

1.1 Cell culture

The mouse breast cancer cell line 4T1 was purchased from ATCC (ATCC number CRL-2539), and the cell line 4T1/luc was constructed by stable expression of firefly luciferase. The 4T1/luc cells showed an equivalent metastatic potential with the parental 4T1 cells. The cells were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% FBS (Gibco), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin, 2 mmol L^{-1} L-glutamine and 400 μg mL^{-1} G418 in a humidified atmosphere of 5% CO_2 in air.

1.2 Transfection and selection of stably transfected 4T1/luc cells

4T1/luc cells at 70%–80% confluence were transfected with 1 μg of luciferase expression plasmid PCAGGS-NEO-luc using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For stable transfection, the cells were exposed to 500 μg mL^{-1} G418 (Gibco) after transfection 1 day later. After growth for 3 weeks, cells were plated at a lower density in RPMI- 1640 with 500 μg mL^{-1} G418 and 10% FBS in 96-wells plates until single colony was formed. Single cloned cells were isolated and expanded. To identify the positive clones, monoclonal cells were plated in 96-well plates and luciferase substrate D-luciferin was added to a final concentration of 60 mg mL^{-1}. After 30 min of exposure, the fluorescence intensity of expression was examined with the optical imaging system (IVIS 200, Xenogen) and the most fluorescent clone was selected and cryopreserved.

1.3 Luciferase activity test of 4T1/luc cells *in vitro*

The 4T1/luc cells were diluted in 96 well plates at a density of 6000, 3000, 1500, 750, 300, 150, and 75 cells per well, respectively, and the cells were exposed to the fluorescent luciferase substrate D-luciferin. The luminous intensity was detected in the camera box by IVIS-Imaging System.

1.4 Cell viability assay

Cell viability was evaluated by using the MTT assay. 4T1/luc cells were seeded in 96-well plates (Costar, USA) at a density of 3×10^3 cells/well in 200 μL medium. The next day cells in triplicate wells were treated with LDM (10^{-14}, 10^{-13}, 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} mol L^{-1}), paclitaxel (10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} mol L^{-1}), or the combination of the two agents. After drug exposure for 48 h, 20 μL MTT solutions (5 mg mL^{-1}) were added to each plate. After being incubated at 37°C for another 4 h, the formazan, which is derived from MTT by living cells, was dissolved in 150 μL per well of DMSO, and the absorbance was detected at 570 nm. The percentage of cell viability was calculated as follows: cell viability (%) = (OD_{570} of experimental well-OD_{570} of blank)/(OD_{570} of control well-OD_{570} of blank). The IC_{50} (defined as the drug concentration with which 50% cell growth was inhibited) was assessed from the dose-response curves. All MTT experiments were performed in triplicate and repeated at least 3 times.

1.5 Western blot analysis

Cells treated with LDM (0.01 nmol L^{-1}), paclitaxel (10 nmol L^{-1}) and the combination of both agents for 48 h were lysed in RIPA buffer on ice and then centrifuged at 12000 r min^{-1} for 10 min at 4°C to collect the supernatant. Protein concentrations were determined using BCA reagent (Pierce, USA). Protein samples were separated by 12% SDS-PAGE and than transferred to a polyvinylidene membrane. After incublated overnight at 4°C in 1% BSA-PBST(phosphate buffered saline contained 0.1% Tween 20), the membranes were incublated with primary and secondary antibodies, band intensity was detected by chemiluminescence using ECL detection reagents (Millipore, USA). Blots were stained with an anti-β-actin to confirm that each lane contained similar amounts of tumor homogenate.

1.6 Wound closure cell migration assay

4T1/luc cells were seeded in a 6-well plate and incublated 24 h to allow for confluency. A 10 μL pipette tip was then used to create a wound through the center of the confluent cell layer, and the size of the wound was measured. Cells were then treated with 3 mL LDM (0.01 nmol L^{-1}), paclitaxel (10 nmol L^{-1}) and the combination of both agents for 24 h. Photos were taken of the wound periodically throughout the assay. To quantify the changes, the size of the wound was measured again after 24 h. This analysis gave the distance (μm) that tumor cells migrated.

1.7 Animal experiments

The 6–8-week-old female BALB/c mice were purchased from the Institute of laboratory Animal Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College. 4T1/luc cells in log phase were suspended to a final concentration of 5.0×10^6 cells mL^{-1} with phosphate-buffered saline (PBS), and 5.0×10^5 4T1/luc cells in 0.1 mL were injected into the second mammary fatpad. Primary tumors were measured every 3 days. When tumors reached about 400 mm^3 in size, the mice were randomized divided into four groups (n = 10 per group). Then the treatment were started, the mice were injected intravenously with LDM (0.05 mg kg^{-1}), intraperitoneally with paclitaxel (5 mg kg^{-1}), and the combination of LDM (0.05 mg kg^{-1}) plus paclitaxel (5 mg kg^{-1}). LDM and paclitaxel were injected once a week and last for two weeks. Control group was injected with saline. Tumor growth was measured with a vernier caliper, and tumor volumes were calculated with the following formula: $V = 0.5a \times b^2$, where a and b are the long and the perpendicular short diameters of the tumors, respectively. At day 30, all mice were weighed and sacrificed, and tumors were excised. Tumors were weighed, and the mean tumor weight was calculated. Tumor growth inhibition was calculated by the following formula: $[(C-T)/C]\times100$ (C, tumor weight of control; T, tumor weight of treated group). The lungs were removed, and fixed in formalin overnight before evaluation of lung metastasis. The number of lung metastases on the lung surface was counted with a magnifying lens.

The tumor growth inhibitory effect was estimated using the treated/control ratio (T/C). In order to calculate the coefficient of drug interaction (CDI), the following equation was used: $CDI=AB/A\times B$, where A or B was the mean T/C of drug A and drug B respectively, and AB was the mean T/C of drug A combined with drug B. A CDI greater than 1 indicates antagonism, a CDI equal to 1 indicates additive and a CDI less than 1 indicates synergy.

1.8 Optical imaging and histological analysis

At day 30, before sacrificed the mice, an optical molecular imaging system was used to evaluate the growth of primary tumor. Luciferase substrate D-luciferin (150 mg kg^{-1}) was injected intraperitoneally, and the animals were placed onto the warmed stage inside the camera box of IVIS-Imaging System (Xenogen) to observe the tumor and the lungs from different groups. Tissues were fixed in 4% paraformaldehyde in PBS, embedded in paraffin and cut into 3–5 μm sections through the center of the tissue specimen. Then the sections were stained with hematoxylin and eosin (H & E). Stained sections were observed using a microscope.

1.9 Statistics

The results were presented as the mean±SD. A one-way analysis of variance was carried out for multiple comparisons. If there was significant variation between treatment and control groups, the mean values were compared by using Student's t-test. P-values less than 0.05 were considered statistically significant.

2 Results

2.1 Luciferase activity in 4T1/luc cells

The bioluminescence photon is positively correlated to the number of cells (Figure 1). The minimum detectable cell number was 750 cells per well according to our results.

2.2 Effect of LDM in combination with paclitaxel on the growth of 4T1/luc breast cells *in vitro*

Dose-response growth inhibitory effects were observed in the MTT assay, and a significant reduction in growth was observes in cells treated with LDM plus paclitaxel compared to cells treated with LDM or paclitaxel alone (Figure 2). And the IC_{50} values of LDM and paclitaxel for 4T1/luc cell were 4.61×10^{-1} and 3.30×10^2 nmol L^{-1}, respectively. LDM showed about 1000-fold much more potent cytotoxicity than paclitaxel.

2.3 Western blot analysis

Western blot analysis was used to detect the metastasis-related and apoptosis-related proteins. As shown, the me-

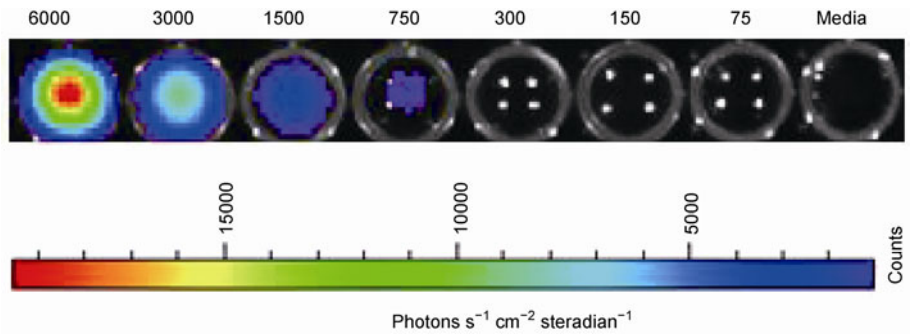

Figure 1 The positive correlation between the photons of bioluminescence and the numbers of 4T1/luc cells.

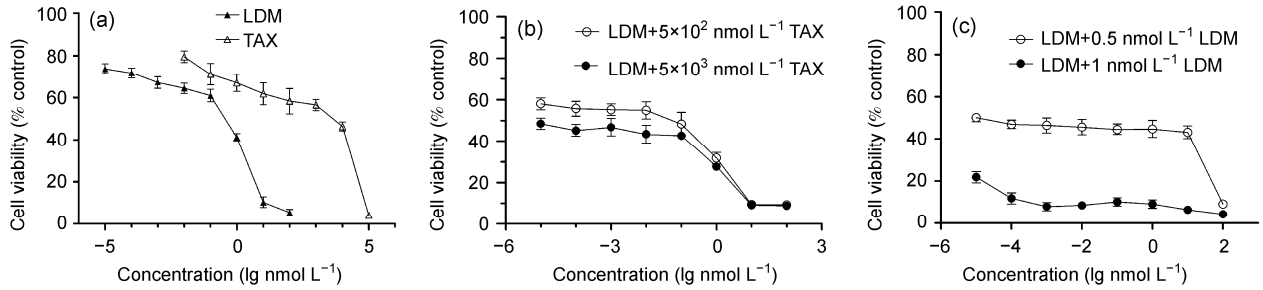

Figure 2 Effect of LDM and paclitaxel on cell viability in 4T1/luc cells. (a) 4T1/luc cancer cells were treated with LDM and paclitaxel alone at concentrations ranging from 10^{-5} to 10^5 nmol L^{-1} for 48 h. (b) and (c) 4T1/luc cells were treated with LDM combined paclitaxel at different concentrations for 48 h.

tastasis-related proteins MMP9, MMP2, VEGF were down-regulated, and the combination treatment had a more obvious down-regulation (Figure 3). The 89 kD cleaved fragment of PARP increased after treated. A markedly enhanced effect was observed in the combination group.

2.4 Wound closure cell migration assay

To examine the effects of LDM, paclitaxel and the combination on tumor cell migration, the cell wound closure assay was performed. In the wound closure assay (Figure 4), the combined group showed a significant (P<0.001) inhibition of cell migration, compared to the LDM group and paclitaxel group.

2.5 Animal experiment

Antitumor and antimetastatic experiments were performed with mouse mammary carcinoma 4T1/luc cell in BALB/c mice. In this study, 4T1/luc tumors were growing rapidly in control group in 30 d during the experiment (Figure 5(a)), and the body weight of each group was recorded (Figure 5(b)). As evaluated on day 30 (Table 1), the LDM and paclitaxel group showed therapeutic efficacy with 38.3% and 36.5% TGI, respectively, and the combination group showed higher therapeutic efficacy with 64.2% TGI for the primary tumor. The CDI value of LDM and paclitaxel was 0.91, indicating synergism.

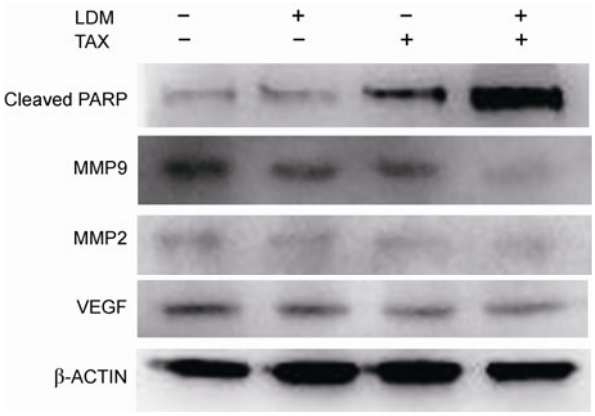

Figure 3 The Western blot analysis of metastasis-related and apoptosis-ralated proteins.

The therapeutic efficacy against metastases was evaluated at day 30 when mice were sacrificed. Lungs were carefully examined. The tumor nodules on the lungs were counted under the dissecting microscope (Figure 6(a) and (c)). Photos of the lungs were taken by IVIS-Imaging System (Figure 6(b) and the pathological changes were observed on sections by HE staining (Figure 6(d)). The combined group significantly reduced the number of lung metastases (Figure 6(b)). The inhibition rates of metastases by LDM, paclitaxel, and the combination of them were 70.2%, 53.8%, and 88.7%, respectively, and the CDI value was 0.82, indicating synergism.

3 Discussion

Metastasis is the most lethal attribute of breast cancer. Once breast cancer metastasizes to distant areas of the body, typically the lung, bones, liver, and central nervous system, it can be treated but is essentially not curable [23]. The 4T1 cell line is widely considered to be one of the best mouse mammary cancer cell lines for the study of human cancer progression [24,25]. This tumor shares many characteristics with human mammary cancers, it closely resembles stage IV human breast cancer, making it an excellent animal model. It was used to investigate inhibitors of DNA-binding proteins necessary for cancer stem cells [26], chemoresistance [27], antimetastatic activities of various drugs and drug combination [28,29]. 4T1/luc cells were engineered for stable expression of firefly luciferase to allow tracking and quantifying of the cells *in vivo*.

As an enediyne anticancer antibiotic, LDM showed extremely potent cytotoxicity toward cultured cancer cells and markedly inhibited the growth of transplantable tumors in mice [30–32]. LDM (C1027) also showed antiangiogenesis and antimetastatic activity, preferentially targeting hypoxic cells [14]. In the previous study, it has been studied for cancer treatment, enhancement of its targeting, selectivity and efficiency, and drug combination therapy of certain cancers [33–35]. As reported, LDM induced unusual DNA double-strand breaks [36]. It could induce apoptosis or mitotic cell death and alter cell cycle progression in many

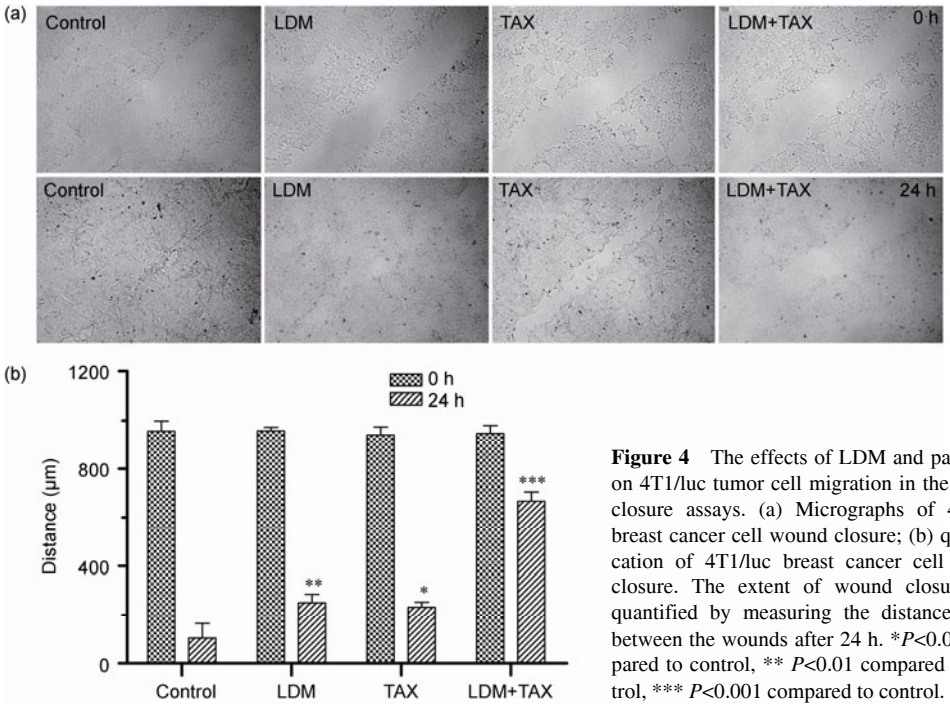

Figure 4 The effects of LDM and paclitaxel on 4T1/luc tumor cell migration in the wound closure assays. (a) Micrographs of 4T1/luc breast cancer cell wound closure; (b) quantification of 4T1/luc breast cancer cell wound closure. The extent of wound closure was quantified by measuring the distances (μm) between the wounds after 24 h. **P*<0.05 compared to control, ** *P*<0.01 compared to control, *** *P*<0.001 compared to control.

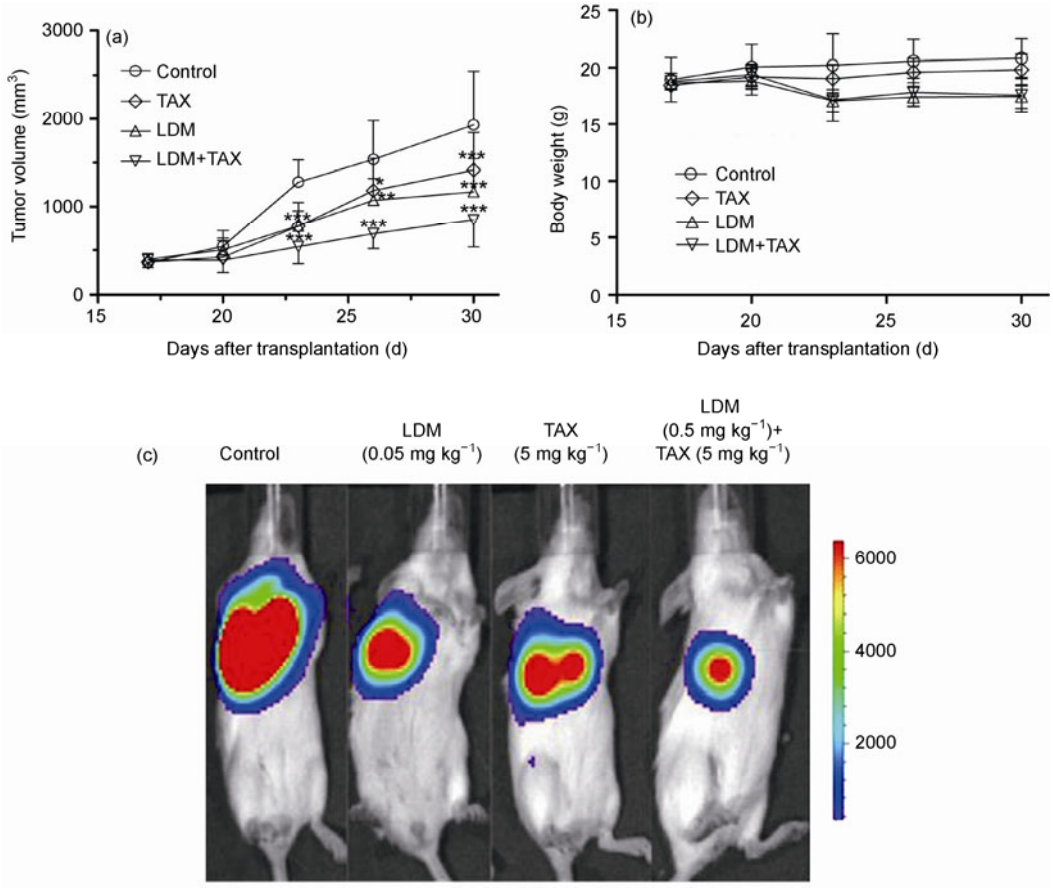

Figure 5 Antitumor activities of LDM, paclitaxel alone or both of them on 4T1/luc breast cancer model. The tumor growth curves (a) and body growth curves (b) in 30 d duration of experiments. Data are expressed as mean ±SD (n=10). (c) Optical imaging in living animal. Color scale represents photons s^{-1} cm^{-2} $steradian^{-1}$, the red dot-cycle indicated the tumor location. * *P*<0.05 compared to control, ** *P*<0.01 compared to control, *** *P*<0.001 compared to control.

Table 1 The growth inhibition of mouse mammary carcinoma 4T1/luc primary tumor in BALB/c mice

Drug	Dose (mg kg^{-1})	Number of mice	Tumor weigh (g) (mean±SD)	Inhibition (%)
Control	–	10/10	5.36±1.05	–
LDM	0.05	10/10	3.31±0.73	38.3[a)]
TAX	5	10/10	3.41±0.80	36.5[a)]
LDM+TAX	0.05+5	10/10	1.91±0.52	64.2[a,b,c)]

a) $P<0.05$ compared to control; b) $P<0.05$ compared to LDM; c) $P<0.05$ compared to TAX.

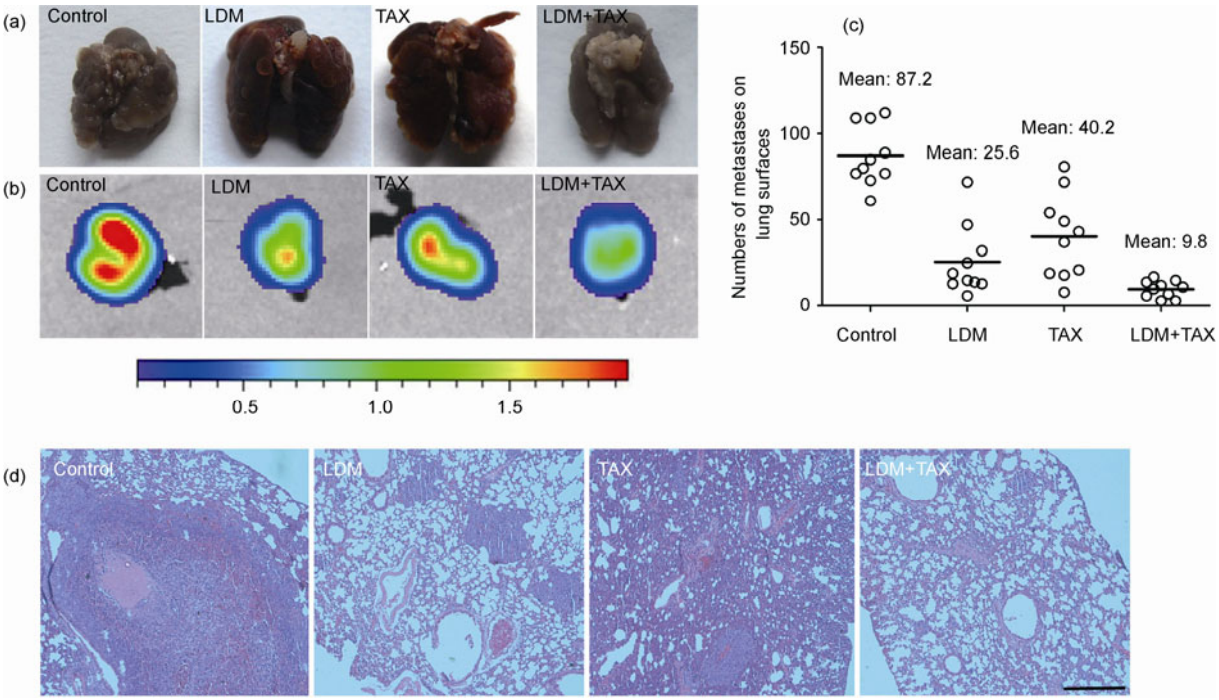

Figure 6 The antimetastatic effect of LDM, paclitaxel, and the combination. (a) Representative photos of the lungs from different groups; (b) optical imaging of lungs from different groups. Color scale represents photons s^{-1} cm^{-2} steradian^{-1}. (c) Metastatic nodules on the lung surface. The number of macroscopic metaststic nodules was observed on the lung surface of treated mice killed on day 30 (n=10). Each data point represents the value for an individual animal. Solid bars represent the mean value for each treatment groups. (d) The pathological changes of lungs by H.E. staining were observed with light microscope (×100).

cancer cells [37]. So far, there are no reports describing the combined efficacy of paclitaxel plus LDM on the antitumor and antimetastatic activities with a breast cancer animal model with highly metastatic potential.

In this study, LDM combined paclitaxel exhibited potent antitumor and antimetastatic effects against the highly metastatic mouse 4T1/luc breast cancer without obvious systemic toxicity. LDM combined paclitaxel resulted an approximately 30% higher inhibition rate in tumor growth compared to that of LDM, paclitaxel alone. The reduction in lung metastases might be due to: (1) LDM and paclitaxel significantly suppressing the growth of the primary tumors. Smaller and less viable primary tumors would beat less risk of dissemination. (2) LDM and paclitaxel directly preventing the formation of the lung metastases. To our knowledge, this is the first report of detection of LDM and paclitaxel combination effected on a metastatic mouse breast cancer model. Evidently, with the construction of the stable expression of firefly luciferase 4T1/luc cell lines, we can monitor and quantitatively analyze the tumor growth and metastasis simply and directly.

In conclusion, LDM or paclitaxel alone exerts antitumor and antimetastatic activity in the highly metastatic mouse breast cancer model. The combination of LDM and paclitaxel synergistically enhances the antitumor and antimetastatic efficacy.

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